SECURITY CLASSIFICATION OF THIS PAGE

REPORT DOCUMENTATION PAGE

1a. REPORT SECURITY CLASSIFICATION Unclassified	1b. RESTRICTIVE MARKINGS
AD-A211 030 JLE ER(S)	3. DISTRIBUTION / AVAILABILITY OF REPORT Approved for public release; distribution unlimited
	5. MONITORING ORGANIZATION REPORT NUMBER(S) AFOSR-TR- 89-1030
6a. NAME OF PERFORMING ORGANIZATION AT & T Bell Laboratories	6b. OFFICE SYMBOL (If applicable)
	7a. NAME OF MONITORING ORGANIZATION Air Force Office of Scientific Research
6c. ADDRESS (City, State, and ZIP Code) 600 Mountain Avenue Murray Hill, NJ 07974	7b. ADDRESS (City, State, and ZIP Code) Building 410 Bolling AFB, DC 20332-6448
8a. NAME OF FUNDING / SPONSORING ORGANIZATION AFOSR	8b. OFFICE SYMBOL (If applicable) NL
	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER F49620-85-C-0009
8c. ADDRESS (City, State, and ZIP Code) Building 410 Bolling AFB, DC 20332	10. SOURCE OF FUNDING NUMBERS PROGRAM ELEMENT NO. 61102F; PROJECT NO. 2312; TASK NO. K2; WORK UNIT ACCESSION NO.

11. TITLE (Include Security Classification)
An investigation into the effects of peptide neurotransmitters and intracellular second messengers in rat central neurons in culture.

12. PERSONAL AUTHOR(S)
John A. Connor

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
Final	FROM 10/84 TO 2/89	6/30/89	7

16. SUPPLEMENTARY NOTATION

17. COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
FIELD	GROUP	SUB-GROUP	intracellular calcium; second messengers; cerebellum; diencephalon; peptides; cAMP; GABA; glutamate; histamine; presynaptic inhibition

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

On continuation pages

20. DISTRIBUTION / AVAILABILITY OF ABSTRACT	21. ABSTRACT SECURITY CLASSIFICATION
☒ UNCLASSIFIED/UNLIMITED ☒ SAME AS RPT ☐ DTIC USERS	unclassified

22a. NAME OF RESPONSIBLE INDIVIDUAL	22b. TELEPHONE (Include Area Code)	22c. OFFICE SYMBOL
Dr. William O. Berry	(202) 767-5021	NL

DD FORM 1473, 84 MAR 83 APR edition may be used until exhausted. All other editions are obsolete.

SECURITY CLASSIFICATION OF THIS PAGE

Final report F49620-85-C-009: Intracellular Second Messengers in CNS Neurons

ABSTRACT

Studies addressing the interrelationship between intracellular messengers and neurotransmitters have been conducted on four types of nerve cell preparations from the mammalian central nervous system and from molluscan neurons using electrophysiological and high resolution digital imaging techniques. Preparations utilized were primary cell cultures from embryonic rat diencephalon and cerebellum, acutely dissociated neurons from the hippocampus of adult guinea pig, and brain slices from the cerebellum of adult guinea pig. The digital imaging methods were largely developed in this laboratory during the course of the contract and the system was one of the first in biology. Major research accomplishments are listed below.

1) First measurements of calcium ion levels in living functional growth cones of mammalian and molluscan neurons and the demonstration of an optimum range of intracellular calcium that promotes outgrowth. It was shown that certain neurotransmitters could modify the path of growth by controlling intracellular calcium. (refs. 1,5,10,11)

2) First reported measurements of membrane conductances in granule neurons of the cerebellum and study of the developmental time course of these conductances. (refs. 2,3)

3) First demonstration of persisting (10 - 20 min) modulation of intracellular calcium levels by brief (1-2 sec) applications of neurotransmitters glutamate and GABA in Purkinje and granule neurons of the rat cerebellum. (ref. 12)

4) First measurements of changes in free calcium levels produced by excitatory amino acid neurotransmitters in the dendrites of hippocampal neurons, a focus of research on memory mechanism. Long lasting changes in intracellular calcium are induced by these agonists that could be one possible basis for changing neuronal responsiveness. (ref. 7)

5) First measurements of calcium levels and oscillations in neurons in the brain slice. Purkinje neurons from adult guinea pig were studied while they were undergoing spontaneous electrical oscillation. (ref. 13)

6) The development of membrane conductances and responsiveness to neurotransmitters and of expression of cell specific antigens has been studied in cerebellar Purkinje neurons in culture and *in vivo*. (refs. 15,16)

SUMMARY OF RESEARCH

CONTROL OF NEURONAL GROWTH CONE MOTILITY BY CALCIUM AND NEUROTRANSMITTERS.

Measurements of calcium ion levels were made in neurons from embryonic rat diencephalon and in neurons from two types of mollusks, *Aplysia* and *Helisoma*.. Electrical activity of the cells was controlled by intracellular microelectrodes and calcium levels measured by digital imaging of fura-2 fluorescence. Cells that were rapidly growing showed high calcium levels in the regions of growth. Where processes had just emerged ffrom the soma or where growth was proceding from more than one pole, calcium levels were uniform and estimated levels of 500 nM were commonly seen. In active growth cones distant from the soma, calcium levels exceeded 200 nM, whereas the soma levels were in the 60 to 80 nM range. Nonextended cells and cells that had stalled in their growth showed uniform calcium levels in the range of 30 - 70 nM. By firing action potentials in the cells and measuring localized 'hot spots' of calcium concentration, it was possible to infer the location of voltage-gated calcium channel in the growth cone membrane.
In cells that expressed receptors for the neurotransmitter serotonin (5-HT), there was a massive intracellular calcium increase to this agonist that caused an arrest of growth.at single stimulated growth cones. Extended periods of action potential firing also caused very large increases in calcium and an arrest of growth. It was shown that there is an optimal range of intracellular calcium that facilitates outgrowth and that excursions above of below this range arrest growth. Acetylcholine, another important neurotransmitter, when applied simultaneously with serotonin prevented the inhibition of neurite elongation that would have resulted from serotonin alone. The results led to a hypothesis that growth cone motioity and neurite elongation can be regulated by voltage and transmitter gated calcium fluses and suggest that the dynamics of neurite morphology may be complexly regulated by and array of neurotransmitters, as is functional electrical activity.

Digital ratio imaging of fura-2 fluorescence was used to determine spatially resolved dynamics of Ca 2+ changes in neuronal growth cones from the mollusks, Helisoma and Aplysia. Time resolution was approximately 1 sec and spatial resolution a few mm depending upon the thickness of the cell region examined. Isolated growth cones of Helisoma were shown to recover from large Ca 2+ loads over a time course of minutes, therefore demonstrating Ca regulation mechanisms not dependent of the rest of the cell. Ca 2+ changes followed during

action potential discharge showed sharply defined spatial gradients within the the growth cones, probably arising from clustering of voltage-gated Ca-channels in the surface membrane. The regions of peak concentration change appeared to shift from central regions to the growth cone periphery as the growth cones matured. There was a marked difference in soma Ca 2+ changes produced by action potentials depending on whether or not the soma had sprouted neurites. Neurite-free somata showed large Ca 2+ changes, whereas in somata that had recently sprouted neurites there were almost no changes for similar electrical stimulation. Measurements on growth cones of N1E115 neuroblastoma cells showed static distributions of Ca 2+ similar to those in the molluscan neurons.

EXCITATORY AMINO ACID RESPONSES IN DENDRITES OF ACUTELY DISSOCIATED HIPPOCAMPAL NEURONS.

Spatio-temporal changes of intracellular the calcium ion (Ca2+) were recorded by digital ratio imaging of fura-2 in pyramidal neurons acutely isolated from the adult guinea-pig hippocampus. Increases in calcium were evoked in tetrodotoxin (5 X 10-7 M) containing saline either by stimulation with excitatory amino acids (EAA's): glutamate or N-methyl-D-aspartate (NMDA) or by depolarization with high potassium (50 mM). Local stimulation with EAA's, applied from a microelectrode as 1-2 sec iontophoretic pulses, at the dendrite induced an increase in intracellular Ca^{2+} predominantly supported by a Ca^{2+} influx at the site of stimulation. The response to the first stimulus generally recovered within one to two minutes. There was no response in Ca^{2+}-free medium (with 1 mM of the chelator EGTA). The response evoked with NMDA in Mg^{2+} free saline was blocked by the specific antagonist APV and was significantly reduced at physiological concentrations of Mg^{2+} (1.8 mM). A second, or in some cases, a third iontophoretic application of an EAA induced a sustained gradient of Ca^{2+}, highest at the site of stimulation, that lasted for periods up to 30 minutes. Agonist was present for only a few seconds. Expression of this extended response usually required at least one priming stimulus by the excitatory agonist. The maintained gradient of Ca^{2+} was supported by a continuous influx of calcium from outside the cell. Fast, short-lasting application of high potassium (50 mM, 5 seconds) set up gradients that collapsed immediately at the end of the stimulus, from which we conclude that conditions of almost unrestricted calcium diffusion inside the cell existed. K-depolarization applied after EAA stimulation produced larger Ca^{2+} changes than the same K stimulus applied before the cell was stimulated with the EAA. Multiple stimuli

with high K alone, induced identical changes in Ca^{2+} and never lead to an extended response. Bath application of GABA (10-100 μM) reduced the magnitude of the maintained Ca^{2+} gradients. The expression of the maintained gradients, but not the immediate response to transmitters was prevented by preincubation of the cells in sphingosine (10 μM), an inhibitor of protein kinase C. We infer a potential role for this kinase in the observed phenomena. The functional significance of the extended response cannot be directly established from these measurements on isolated neurons, but its properties could give rise, in part, to mechanisms involved in neural plasticity as observed in long term potentiation or in kindling epileptogenesis.

RESPONSES OF PURKINJE NEURONS TO EXCITATORY TRANSMITTERS DURING DEVELOPMENT

Calcium ion levels in cerebellar Purkinje neurons, in culture, have been measured using the fluorescent indicator, fura-2, and digital imaging. Cells were loaded with the indicator both by injecting the free acid form and by allowing the membrane permeant form (/AM) to become deesterified and trapped. The two methods gave significantly different results in that the /AM loaded cells showed localized regions of high Ca 2+ in the soma whereas the injected cells did not. Resting levels in the remainder of the cytoplasm were similar however, as were the excursions in Ca2+ induced byelectrical or chemical stimulation. Comparison of the data from the two methods suggests that qualitative measures of Ca in intracellular stores can be derived from the /AM loading method. Injected cells generally showed high Ca^{2+} levels in the soma that persisted for 3-8 minutes following removal of the injection electrode. The dendrites of these cells however maintained low Ca 2+ levels and differences of several hundred nM in Ca^{2+} were maintained between the soma and initial dendrite segment, demonstrating directly the large Ca pumping capacity of the dendrites. Localized regions of high Ca^{2+} in dendrites could be generated by applying glutamate from a microelectrode in TTX-Krebs saline. When studied in culture media with 4.7 mM K, the Purkinje neurons showed a bimodal distribution of Ca 2+ with 35 to 40% showing stable Ca^{2+} levels between 250 and 350 nM, and the remainder 80 to 130 nM Ca^{2+}. Granule neurons on the same coverslips had Ca^{2+} level in the lower range in >95% of the examples observed. Stimulus of the low Ca^{2+} Purkinje neurons with 1-3 s iontophoretic applications of glutamate from an extracellular microelectrode triggered increases in intracellular Ca^{2+} that lasted for periods of several minutes. Similar Ca^{2+} changes induced by K depolarizations recovered in less than 1 min. It was concluded that the neurotransmitter was capable of

driving the Purkinje neurons into a metastable state in which Ca^{2+} levels could remained elevated for long periods and hence calcium dependent intracellular processes, activated or inactivated for long periods following brief stimulus.

CALCIUM ION CHANGES IN CEREBELLAR PURKINJE NEURONS IN BRAIN SLICE.

Microfluorometric imaging was used to study the correlation of intracellular calcium concentration with voltage-dependent electrical activity in guinea pig cerebellar Purkinje cells. This was the first such study if its kind capable of such spatial and temporal resolution of absolute Ca levels. The spatiotemporal dynamics of intracellular calcium concentration were demonstrated during spontaneous and evoked activity. The results supported hypotheses of dendritic segregation of calcium conductances suggested by electrophysiological experiments. These *in vitro* slice fluorescence imaging methods are applicable to a wide range of problems in central nervous system biochemical and electrophysiological functions.

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PERSONEL ASSOCIATED WITH PROJECT

John A. Connor, PhD (principal investigator)	10/84-1/89
Phillip E. Hockberger, PhD.	10/84-9/87
Hillel Chiel, PhD	9/85-8/87
Zahur Ahmed, PhD	10/84-12/84
Wytse Wadman, PhD	5/87-6/87; 4/88-7/88
Alan Kay, PhD	4/88-1/89
Kenneth McMillan	5-/85-6/85

John A Connor
7/5/89